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Optimization of cultural and nutritional conditions for carboxymethylcellulase production by *Aspergillus hortai*

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ABSTRACT

The potential production of carboxymethylcellulase (CMCase) by *Aspergillus hortai* in liquid state fermentation was studied. Cultural and nutritional factors affecting CMC production were also investigated in order to optimize the fermentation conditions for the maximization of production. The obtained results revealed that, the maximum CMCase production (0.23 U/ml) was achieved after 96 h in a liquid medium (PH7) inoculated with 10% v/v, at temperature 37 °C, containing (g L^{-1}) CMC, 5.0; yeast extract, 0.1; $(NH_4)SO_4$, 0.5; KH_2PO_4, 10.0; $MgSO_4 \cdot 7H_2O$, 0.1 and NaCl, 0.2. and the activity remained almost stable between pH 6 and 7. The highest CMCase activity (1.18 U/ml) was obtained at a lactose concentration of 5.0 g L^{-1}.

1. Introduction

Cellulase is an industrial enzyme, which is mainly produced by fungi and bacteria. It is responsible for cellulose degradation by catalyzing the hydrolysis of β-1, 4 glycosidic bonds in cellulosic materials, to produce short cellulo-oligosaccharides and glucose (Afsahi, Kazemi, Kheirolomoom, & Nejati, 2007; Xu et al., 2007). Cellulases (1,4-β-D-glucanglucanohydrolase, EC 3.2.1.4) are multienzyme complexes, comprising three main components; endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21), which have been shown to act synergistically in the hydrolysis of cellulose to glucose unit (Emert, Gum, Lang, Liu, & Brown, 1974; Ryu & Mandels, 1980; Sim & Oh, 1990).

Biotechnology of cellulases began in early 1980s first in animal feed followed food applications. During the last two decades, the use of cellulases has increased considerably, especially in textile, pulp and paper industries (Bhat, 2000). Researchers have strong interests in cellulases because of their applications in various industries, including starch processing, grain alcohol fermentation, brewery and wine,

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extraction of fruit and vegetable juices, textile, detergents, animal feed, pulp and paper, as well as in research development (Gao et al., 2008 and Zhou et al., 2008).

Cellulases are synthesized and produced by several microorganisms including bacteria, actinomycetes and fungi, but the latter are of great interest because they excrete their enzymes extracellularly (Bollok & Reczey, 2005; Immanuel, Dhanusa, Prema, & Palavesam, 2006). *Trichoderma reesei* is the most efficient producer of endo- and exo- glucanases (Miettinen-Oinnonen & Suominen, 2002), but does not excrete a sufficient amount of β-glucosidase (Bollok & Reczey, 2005) for which *Aspergillus* strains are known to be good producers Juhasz, Kozma, Szengyel, and Reczey (2003). Jahangeer et al. (2005) found that the cellulase producing fungi belonged to *Aspergillus* species, *Trichoderma* sp., *Fusarium* sp., *Alternaria* sp., *Pencillium* sp. and *Rhizopus* sp. Bansal, Tewari, Soni, and Soni (2012) reported that cellulases were also produced by *Aspergillus niger* NS-2 in solid state fermentation on agriculture and kitchen waste residues.

Cellulase appear to depend on a complex relationship involving a variety of factors like inoculum size, carbon source and cellulose quality, pH value, temperature, presence of inducers, medium additives, aeration, growth time, etc. (Immanuel et al., 2006 and Iqbal, Ahmed, Zia, & Irfan, 2011). Therefore, attention has been focused on studying the cellulytic activity and cellulase enzyme production by several microorganisms in various products as well as in various environments. To establish a successful fermentation process it is necessary to make the environmental and nutritional conditions favorable for the microorganism for overproduction of the desired metabolite.

This work was undertaken to investigate the optimization of extracellular carboxymethylcellulase production from *Aspergillus hortai* strain in submerged culture and according to our knowledge this is the first research for production of cellulase by *A. hortai*.

2. Materials and methods

2.1. Organism and culture conditions

A. hortai strain was provided by the center of culture collection, National Research Center (NRC), Egypt. The slants of *A. hortai* were incubated in potato dextrose-agar medium (PDA) at 30 °C for 7 days. A spore suspension of about 5.2×10^8 spores/ml was prepared in sterile distilled water containing about 0.01% (v/v) Tween 80. This solution was used as a source of inoculums (Shaibani, Ghazvini, Andalibi, & Yaghmaei, 2011).

2.2. Cellulase production

Two ml of standard inocula of *A. hortai* were inoculated in 250 ml-Erlenmeyer flask containing 50 ml of sterilized carboxymethyl cellulose (CMC) synthetic liquid medium containing (g L^{-1} of distilled water) CMC 1.1, yeast extract 0.1, $(NH_4)_2SO_4$0.5, $KH_2PO_4$10.0, $MgSO_4.7H_2O$ 0.1, NaCl 0.2. The pH of the medium was adjusted before sterilization to 5.0. The inoculated flasks were incubated on orbital shaker (150 rpm) at 28 ± 2 °C for 3 days. Liquid state culture was centrifuged at 8000 rpm for 20 min at 4 °C (Ali et al., 2009). The resulting supernatant was called as crude enzyme preparation.

2.3. Assay of carboxymethylcellulase activity

CMCase (endoglucanase) activity was assayed by determination of reducing sugar (glucose) released from carboxymethyl cellulose (CMC) as a substrate. The culture supernatant (0.5 ml) was incubated with 0.5 ml 1% CMC in 0.05 μM sodium acetate buffer solution, (pH 5.0 ± 2) at 40 °C for 1 h. The resulted reducing sugars were determined according to Miller (1959) by dinitrosalicylic acid (DNS) using glucose as standard. Enzyme activity was determined in terms of international unit (U) which is define as the amount of enzyme required to liberate one μ mole of glucose per minute.

2.4. Optimization of fermentation parameters

In these experiments, the conditions for cellulase production by *A. hortai* were optimized. Three replicate were used in each determination and values were recorded as the mean of the three replicateœ ± standard deviation.

2.4.1. Time course

Standard inocula (5.2×10^8 spores/ml, 4% v/v) was inoculated in each 250 ml-Erlenmeyer flask containing 50 ml of CMC broth medium (pH 5). The inoculated flasks were placed in a shaker (150 rpm) at 30 °C for 7 days. Samples were collected after 24, 48, 72, 96, 120, 144 and 168 h of incubation for cellulase activity. Enzyme activity was measured by dinitrosalicylic acid (DNS) as mentioned before.

2.4.2. Inoculum size

Aliquots (50 ml) of the production medium inoculated with different inoculum size (2, 4, 6, 8, 10, 14% v/v) of the selected *A. hortai*. At the end of incubation period, cellulase activity was assayed.

2.4.3. Culture age

Aliquots (50 ml) of the production medium inoculated with two ml of standard inocula collected after 3, 4, 5, 6 and 7 days of the selected *A. hortai*, and then the fermentation studies were carried out and the cellulase activity was assayed.

2.4.4. pH

The pH of the media was optimized by using two buffer systems (phosphate buffer and acetate buffer). The pH was set at different levels (4.5, 5, 5.5, 6, 6.5, 7, 7.5 and 8) using the buffers. The optimum inoculum size determined before was inoculated in 250 ml-Erlenmeyer flasks containing 50 ml of fermenting medium. The flasks were placed in a shaker (150 rpm) at 30 °C for the optimum time determined before, then CMCase activity was determined.

2.4.5. Fermentation temperature

The fermentation medium was adjusted at the obtained optimum pH value and inoculated with the obtained optimum inoculum size. The inoculated flasks were incubated in an orbital shaker at the optimum agitation at different temperatures (25, 30, 37, 40 and 50 °C) for optimal incubation time. At

the end of incubation period, the enzyme activity was assayed.

2.4.6. *Fermentation medium*

For identifying the optimum fermentation medium for *A. hortai* four different media were used.

I. Czapek's Dox liquid medium containing (g L^{-1}): CMC as a sole carbon source, K_2HPO_4, 1; yeast extract, 5; CMC, 10; Czapek's concentration, 10 ml Coral, Arikan, Ünaldi, and GÜvenmez (2002).
II. CMC liquid medium containing (g L^{-1}): CMC, 1.0; yeast extract, 0.1; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 10.0; $MgSO_4.7H_2O$, 0.1 and NaCl, 0.2. Kocher, Kalra, and Banta (2008).
III. CMC liquid medium containing (g L^{-1}): CMC, 5.0; yeast extract, 0.1; $(NH_4)_2SO_4$, 0.5; KH_2PO_4, 10.0; $MgSO_4.7H_2O$, 0.1 and NaCl, 0.2.
IV. Peptone cellulose medium containing (g L^{-1}): peptone, 5.0; cellulose, 10.0; yeast extract, 1.0; $CaCO_3$, 3.0 and NaCl, 5.0.

Each medium was adjusted at the optimum pH and inoculated with the optimum inoculum size. The inoculated flasks were placed at the optimum agitation rate at the optimum temperature for the optimum time. At the end of incubation time, the CMCase activity was assayed.

2.4.7. *Different carbon sources*

For optimization of carbon sources of the fermentation medium different carbon sources; glucose, fructose, maltose, sucrose, lactose, galactose, arabinose and starch (5 g/l) were separately added as a sole carbon source. The optimized media were incubated in an orbital shaker at the optimum agitation rate (150 rpm). At the end of incubation time the enzyme activity in cell-free culture supernatant was assayed. Carbon sources were autoclaved separately and added to the medium under aseptic conditions (Jahangeer et al., 2005).

2.4.8. *Optimization of nitrogen source*

CMC liquid fermentation medium was separately supplemented with (2.5 g/l) urea, ammonium sulfate, ammonium nitrate, ammonium chloride, yeast extract, beef extract, peptone, sodium nitrite, ammonium dihydrogen phosphate as a nitrogen source was studied.

2.4.8.1. *Statistical analysis*. The significance of the data with different factors was evaluated using one-way analysis of variance ANOVA. All analyses were performed with SAS Software package version 6.12 (SAS, 1997).

3. Results and discussion

3.1. *Fermentation time course*

For optimization of incubation time, the inoculated flasks of fermentation medium were incubated at different times ranging from 24, 48, 72, 96, 120, 144 and 168 h. Table 1 shows that CMCase activities were 0.005, 0.026, 0.048, 0.06, 0.057, 0.056 and 0.055 U/ml, respectively. These values indicated that the highest CMCase activity was achieved after 96 h of the fermentation time. Almost, similar results have been found by Acharya, Acharya, and Modi (2008) who found that cellulase activity by *A. niger* using saw dust as substrate were 0.028, 0.033, 0.04, 0.096, 0.052, 0.049, 0.038 and 0.015 U/ml after 24, 48, 72, 96, 120, 144, 168 and 192 h, respectively.

Table 1 – Effect of fermentation time course on carboxymethyl cellulose production by liquid state culture of *A. hortai*.

Time (hour)	Activity (U/ml)
24	0.005 ± 0.001^{D}
48	0.026 ± 0.002^{C}
72	0.048 ± 0.006^{B}
96	0.061 ± 0.0015^{A}
120	0.057 ± 0.0035^{AB}
144	0.056 ± 0.0035^{AB}
168	0.055 ± 0.0025^{AB}

3.2. *Effect of inoculum size*

Inoculum size of the fungal culture has an important effect on CMCase production. In order to improve the activity of CMCase, different amounts of standard inocula (2, 4, 6, 8,10 and 14%, v/v) were inoculated into liquid state fermentation medium. Fig. 1, shows that inoculum size of 10% v/v gave the maximum CMCase activity (0.083 U/ml). The results of Iqbal, Asgher, Ahmed, and Hussain (2010) confirmed our results, where they observed maximum CMCase productivity from *Trichoderma harzianum* at 10% inoculum size. Whereas, Garg and Neelakantan (1981) observed the highest CMCase production by *Aspergillus terrus* at 5.0% v/v inoculum size.

Lower cellulase biosynthesis at lower inoculum size is probably due to less conidial cells which are insufficient to use the fermentation medium for enzyme maximal activity, while the decreased yield at higher inoculum size is probably due to nutritional imbalance caused by tremendous growth resulting in autolysis of cells (Haq, Iqbal, & Qadeen, 1993).

3.3. *Culture age*

From Fig. 2, it was clearly evident that, cellulase activity proved to be the enzymic reaction dominating at all the growth

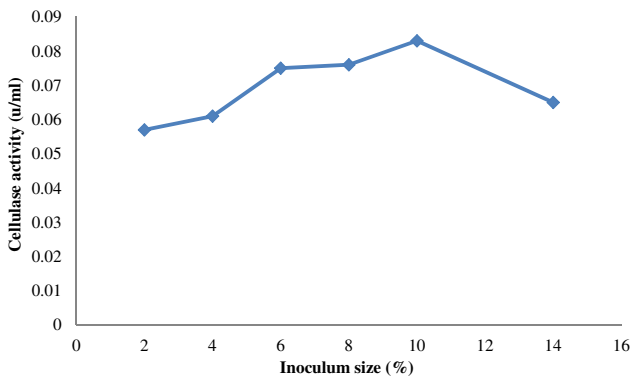

Fig. 1 – Effect of inoculum size on CMCase production by *A. hortai* in liquid state fermentation.

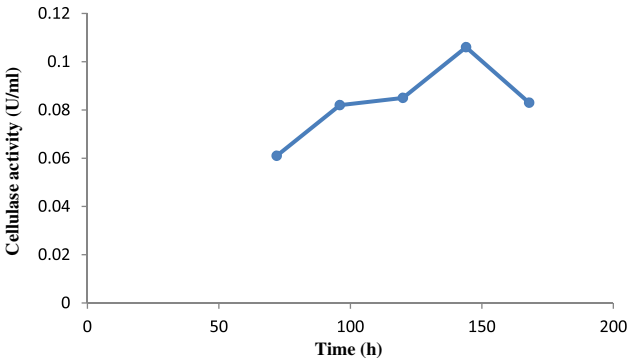

Fig. 2 – Effect of time course of cultivation on CMCase production by *A. hortai* in liquid state fermentation.

phases (72–168 h) of *A. hortai*. However, comparatively higher yield of cellulase (0.16 U/ml) were formed in the stationary phase of the growing cells 144 h (Fig. 2). Similar results were reported by Romero, Aguado, Gonzalez, and Ladero (1999).

3.4. *pH Value*

Generally, enzymes have an optimum pH value at which their activity is highest and at higher or lower pH values, their activity decreases (Lehinger et al., 1993). The pH value of the fermentation medium for cellulase production by fungi is considered the most important factor. CMCase production by *A. hortai* in liquid state fermentation was examined at various buffered pH values ranging from 4.5 to 8.0. The maximum CMCase activity (0.160 U/ml) was observed at pH 7 (Fig. 3). This value is in accordance with Akiba, Kimura, Yamamoto, and Kumagai (1995) who reported that the optimum pH for cellulase production by *A. niger* was between 6.0 and 7.0. The results obtained in our study are also in agreement with Gautam et al. (2011) who observed the highest production of cellulases by *A. niger* and *Trichoderma* sp. at pH 6.5. Also, Bansal et al. (2012) found the highest cellulases production by *A. niger* NS-2 was at pH 7.0.

3.5. *Optimum temperature*

The incubation temperature of the fermentation medium is one of ultimate factor influencing the production of enzymes.

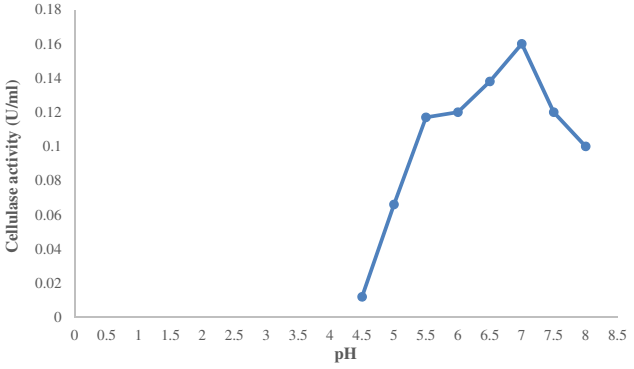

Fig. 3 – Effect of pH on CMCase production by *A. hortai* in liquid state fermentation.

Enzymes have an optimum temperature at which their activity is maximum and at higher or lower temperatures, their activity decreases. Optimization of temperature was achieved by incubation the inoculated flasks containing fermentation medium at different temperatures (25, 30, 37, 40 and 50 °C). The results of CMCase activity were illustrated in Fig. 4.It is obvious that the maximum CMCase activity was observed at 37 °C (0.20 U/ml) and CMCase activity at 30 °C was mediated (0.16 U/ml). These results agreed well with the results of Jahangeer et al. (2005) who found that some *Asperillus* species had high degree of enzyme production at 37 °C. Some investigators found maximum cellulase production at higher temperatures and others recorded highest yield at lower temperatures in comparison with our results. Ali, Sayed, Sarker, and Alam (1991) reported a maximum yield of cellulase from *A. terrus* at 40 °C on water hyacinth after 6 days. Immanuel, Bhagavath, Iyappa Raj, Esakkiraj, and Palavesam (2007) found high level of cellulase production at 40 °C (0.292 and 0.258 U/ml) by *Aspergillus fumigatus* and *A. niger* using coir waste.

Bansal et al. (2012) reported that *A. niger* NS-2 exhibited a wide range of temperature for its growth and cellulase production on agriculture and kitchen waste residues. Highest yields of CMCase, FPase and β-glucosidase were obtained at 30 °C after 96 h. The optimum temperature for cellulase production by *A. fumigates* was reported to be 32 °C (Gilna & Khaleel, 2011).

3.6. *Fermentation media*

Four different fermentation media were used to identify the optimum fermentation medium for CMCase production by *A. hortai*. Fig. 5 shows that the highest CMCase production (0.23 U/ml) was obtained when *A. hortai* was grown in CMC liquid medium containing 5 g of CMC/liter (medium III) with lactose as an inducer. These results agreed well with the results of Ahmed and Vermette (2008) who study the production level of cellulase enzyme in *T. reesei* RUT-C30 strain using four medium compositions, *T. reesei* strain grew very well in cellulose–yeast extract medium with lactose as an inducer.

3.7. *Effect of different carbon source*

For optimization of carbon sources, *A. hortai* was grown in fermentation medium (CMC liquid medium) containing various carbon sources. The carbon sources (5 g/l) used were

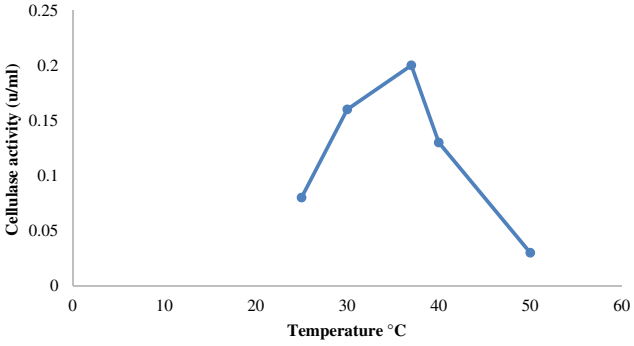

Fig. 4 – Effect of temperature on CMCase production by *A. hortai* in liquid state fermentation.

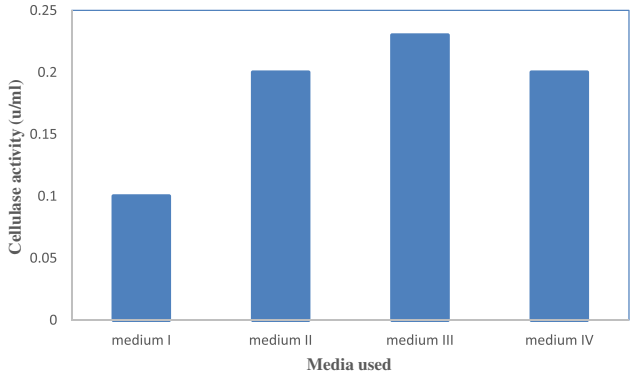

Fig. 5 – Effect of different media on CMCase production by *A. hortai* in liquid state fermentation.

glucose, fructose, maltose, sucrose, lactose, galactose, arabinose and starch which were separately added to the fermentation medium for maximum CMCase production. The results in Fig. 6 indicates that the maximal cellulase activity (1.18 U/ml) was observed when lactose used as carbon source, and the lower CMCase production was recorded when glucose, galactose or starch were used as carbon sources, this result is agreed with Ahmed, Bashir, Saleem, Saadia, and Jamil (2009) where he found that carboxymethyl cellulose (CMC) induced cellulase production by *T. harzianum* whereas glucose repressed the synthesis of cellulase. This might be due to the lactose induce the enzyme activity, or may increasing the penetration rate of lactose through the cell membrane (Miyamoto, Ooi, & Kinoshita, 2000).

3.8. *Effect of different nitrogen sources*

To detect the appropriate nitrogen source for cellulase production by *A. hortai*, the fermentation medium was supplemented with five inorganic (ammonium sulfate, ammonium nitrate, ammonium chloride, sodium nitrite and ammonium dihydrogen phosphate) and four organic (urea, yeast extract, beef extract and peptone) nitrogen sources. The nitrogen sources (2.5 g/l) used were separately added to the fermentation medium for maximum CMCase production. The results in Fig. 7 indicates that peptone caused maximum CMCase

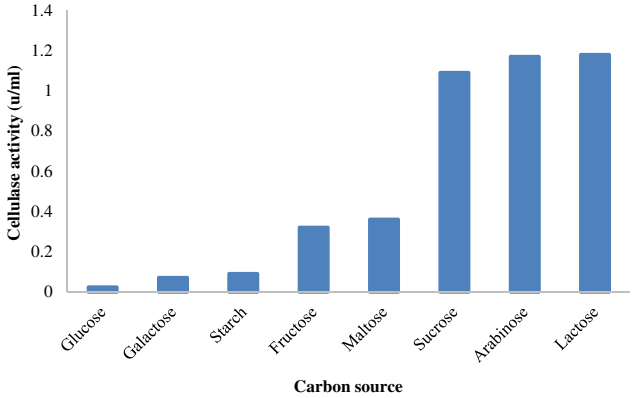

Fig. 6 – Effect of carbon source on CMCase production by *A. hortai* in liquid state fermentation.

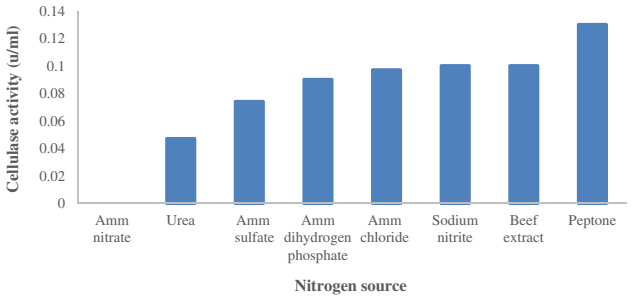

Fig. 7 – Effect of nitrogen source on CMCase production by *A. hortai* in liquid state fermentation.

production (0.13 U/ml) and generally it was observed that organic nitrogen sources gave better CMCase activity than inorganic nitrogen sources. These results agreed with the results of Deswal, Khasa, and Kuhad (2011) who found that urea caused maximum CMCase production and inorganic nitrogen sources did not exhibit any significant effect on increase in enzyme production. On the contrary, Sasi, Ravikumar, and Kani (2012) found that *Aspergillus flavus* showed the highest production of cellulase enzyme utilizing ammonium sulfate as nitrogen source than yeast extract. Kocher et al. (2008) reported that the best nitrogen source of *T. harzianum* MTCC 8230 when grown on rice straw was $(NH_4)_2SO_4$(0.5 g L^{-1}) as nitrogen source.

4. Conclusion

This work studied the bioconversion of cellulose by cells of *A. hortai* observed to be isolated from soil. Optimization of the fermentation conditions for the maximization of production of cellulose were studied. Parameters affecting bioconversion of cellulose namely time course, inoculum size, *pH value*, optimum temperature and fermentation media were carried out. The highest CMCase production (1.18 U/ml) was obtained when *A. hortai* was grown in CMC liquid medium containing 5 g of lactose/liter as a carbon source. The previously mentioned achievements may contribute to the improvement of the biotechnology basis of cellulose bioconversion to the industrial importance of cellulose.

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